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Humanized Mouse Models for Epstein Barr Virus Infection

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Short title: EBV in humanized mice

Abstract

It is essential for the human immune system to control Epstein Barr virus (EBV), because this common human γ-herpesvirus efficiently spreads through the human population with more than 90% being persistently infected after 20 years of age even in developed countries. Moreover, it threatens each host with its potent growth transforming properties, readily immortalizing human B cells into persistently growing lymphoma cell lines. Since this virus only infects humans, mice with reconstituted human immune system components provide an informative in vivo model to study EBV infection, the associated tumor formation and immune control thereof. They recapitulate the different infection programs in human B cells, allow modeling EBV driven lymphoma formation and interrogation of the key cytotoxic lymphocyte responses that are also required to control this pathogen in humans. The respective lessons that were taught by these investigations will be discussed in this review as well as the challenges in the future to address the whole portfolio of EBV associated diseases and how they could be prevented by EBV specific immunotherapies.

1. Introduction to EBV (500 words)

Epstein Barr virus (EBV) is one of the most successful pathogens in the human population, establishing persistent infection in more than 90% of the human population, and at the same time the most potent human tumorvirus, readily transforming human B cells in culture into lymphoblastoid cell lines (LCLs) [1]. Despite this strong growth transforming ability, fortunately, only a small group of persistently EBV infected individuals develop tumors that are associated with this virus [2]. These are mainly malignancies of epithelial and B cell origin, like nasopharyngeal carcinoma and Hodgkin as well as Burkitt lymphoma, respectively. The EBV associated B cell lymphomas express different sets of EBV latent antigens that are in their majority not expressed during infectious virus production, so called lytic EBV replication. In the latency I pattern that is found in Burkitt's lymphoma and homeostatically proliferating memory B cells of healthy virus carriers, only the EBV nuclear antigen 1 (EBNA1) is expressed at the protein level [3]. In the 40% of Hodgkin's lymphoma that are EBV associated, EBNA1 and the two latent membrane proteins (LMP) 1 and 2 are expressed. This latency II expression pattern can be found in germinal center B cells of healthy EBV carriers [4]. Finally in immunoblastic lymphomas, like diffuse large B cell lymphomas, all 8 latent gene products (EBNA1, 2, 3A, 3B, 3C, LP and LMP1, 2) are expressed. Naïve B cells of healthy EBV carriers also display this latency III infection program. Thus, healthy EBV carriers harbor all EBV latency programs that can also be found in virus associated B cell lymphomas and the pro-proliferative as well as pro-survival functions of these latency patterns are thought to allow EBV to differentiate infected cells into memory B cells, the site of long-term EBV maintenance [5] and of reactivation into lytic replication upon plasma cell differentiation [6].

The immune system is thought to stand between established latent EBV infection and tumor formation in healthy EBV carriers [1]. Indeed, immune suppression by therapy after transplantation or due to human immunodeficiency virus (HIV) coinfection leads to an increased incidence of EBV associated malignancies [7]. Primarily cytotoxic lymphocytes are thought to control EBV infection and prevent tumor formation, because post-transplant lymphoproliferative diseases (PTLD) can be efficiently treated by adoptive transfer of EBV specific T cell lines [8] and primary immunodeficiencies that often selectively predispose for uncontrolled EBV infection primarily affect natural killer (NK) and $CD8^+$ T cell development, stimulation or function [9••]. However, in order to interrogate these cytotoxic lymphocyte populations for their efficacy to control EBV infection and the portfolio of EBV associated malignancies as well as, furthermore, to induce them with vaccination approaches, an in vivo model of EBV infection, tumorigenesis and immune control is crucial. The search for such a model was complicated by the exclusive tropism of EBV for humans and the absence of close relatives of this virus outside of old world

monkeys [10]. Since ten years such a model based on mice with reconstituted human immune system components (HIS mice) is being explored [11] and this review will summarize our understanding of which aspects of EBV immunobiology can be modelled in HIS mice and which new aspects of EBV infection, tumorigenesis and immune control this model has revealed.

2. Modelling EBV infection (500 words)

As discussed above, EBV infection is a finely orchestrated set of infection programs that allow the virus to differentiate its infected host cells into memory B cells for long term persistence and reactivate from this reservoir at mucosal surfaces for shedding into the saliva with the purpose of transmission to new hosts [12]. His mice recapitulate the different EBV infection programs in B cells, but cannot so far serve as a model of the final lytic replication in oropharyngeal epithelial cells, which the virus uses to amplify infectious virus production during shedding into the saliva [13-15]. Within the B cell compartment, latency III predominates after infection in currently available HIS mouse models, namely NOD-*scid* $\gamma_c^{-/-}$ or BALB/c Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice with reconstituted human immune system compartments from human CD34$^+$ hematopoietic progenitor cells (huNSG or huBRG) with or without an fetal liver plus thymus organoid implanted under the kidney capsule (BLT) [16-21•]. However, lytic infection and the lower latencies of EBV can also be detected [20-22•]. While most of these studies have been conducted by immunohistochemistry and absence of latent EBV protein detection in the presence of non-translated EBV RNAs, like EBERs, can be difficult to interpret, alternative promotor usage for EBNA1 (Q promotor usuage), which occurs during latency I and II has been detected in huNSG mice [22•]. Interestingly, Qp usage for EBNA1 transcription seemed to be dependent on CD4$^+$ helper T cell presence, which is also required for germinal center B cell survival. Therefore, the authors suggested that as in human healthy EBV carriers, latency II infection can only be accessed in germinal center B cells with the help of presumably follicular helper T cells [23]. However, conditions, under which these lower latencies can be assessed in HIS mice need to be characterized in more detail in the future. Similarly, lytic EBV replication does seem to be well controlled in HIS mice after infection with the prototypic B95-8 EBV strain, originally isolated from an American patient with symptomatic primary EBV infection (infectious mononucleosis or IM) [24,25]. Expression of the immediate early EBV transcription factor BZLF1 is only rarely detected in spleen sections of infected HIS mice [26•-28]. Comparing infections with wild-type (wt) or BZLF1 deficient B95-8 EBV a difference in peripheral blood viral loads was only detected three weeks after infection and viral titers were quite heterogenous at this timepoint for wt EBV infection [28]. These findings suggest that EBV only significantly reactivates into lytic replication from a latent infection after two weeks of primary infection and that lytic replication is quite efficiently controlled by cytotoxic lymphocytes at week four after infection, as discussed below. Moreover, EBV viral loads plateau or peek at four to six weeks after primary infection of HIS mice with a kinetic that is quite similar to symptomatic primary EBV infection in humans [29•].

These characteristics can be altered by using different EBV virus isolates or mutant viruses. Along these lines, the M81 EBV strain, isolated from an Asian nasopharyngeal

carcinoma patient, and related isolates reactivate lytic EBV replication more readily than B95-8 EBV and this might be connected to polymorphisms in their BZLF1 gene [27,30]. Such viral strains might be more informative to assess the role of lytic EBV antigens after infection in HIS mice. However, viral mutants have so far been mainly assessed on the B95-8 strain background. These include EBNA3B, LMP1, LMP2, BZLF1 and EBER deficient EBV strains [21•,26•,28,31•-34]. Surprisingly, all of these viruses established persistent EBV infection in HIS with often surprising effects on EBV associated lymphoma formation, as will be discussed in the next chapter.

3. Modelling EBV associated tumorigenesis (500 words)

As for the infection only the B, but not the epithelial derived tumorigenesis can so far be studied in EBV infected HIS mice. The tumors that develop upon infection with hundred thousand infectious viral particles of EBV in 20-30% of HIS mice are mostly associated with spleen, mesenteric lymph node, kidney and liver, sometimes with effusions into the peritoneal cavity [18,26•,28,31•]. They consist primarily of latency III EBV infected B cells with inflammatory infiltrates of T cells, reminiscent of post-transplant lymphoproliferative disease [18,26•,31•]. Interestingly, these EBV associated lymphomas are less efficiently established in the absence of lytic EBV infection [21•,28]. This could indicate that an amplification of B cell infections by infectious virus particle production leads to enhanced lymphomagenesis. However, in the respective studies mainly early, but not late lytic EBV antigens could be detected by immune histochemistry [21•,35]. Therefore, the respective authors suggested that abortive lytic replication produces paracrine factors that are beneficial for lymphoma development. Indeed, injection of lytic cycle competent or deficient EBV transformed cell lines into mice without human immune system compartments led more frequently to tumors of lytic cycle competent cells, and these could not be inhibited by blocking infectious virus production with acyclovir [36]. In addition to the EBV lytic cycle, deficiencies in three of eight latent EBV proteins and absence of the non-translated EBER RNAs have been tested during EBV infection of HIS mice. While EBER deficiency did not alter EBV infection [34], EBNA3B deficiency surprisingly led to increased tumorigenesis [31•]. The resulting lymphomas presented with decreased inflammatory infiltrates and, therefore, appeared histologically like EBV associated diffuse large B cell lymphomas (DLBCLs). Their transcriptional profile resembled three cases of EBNA3B deficient DLBCLs from patients with a marked downregulation of the proinflammatory chemokines CXCL9 and 10. Expression of CXCL10 in EBNA3A deficient EBV transformed B cells restored T cell mediated immune control. Thus, EBNA3B seems to be a viral tumor suppressor that prevents premature death of the persistently EBV infected host due to tumor formation. The two latent membrane proteins LMP1 and 2, especially 1, are considered essential oncogenes of EBV driving proliferation and apoptosis resistance by mimicking CD40 and B cell receptor signaling, respectively [12]. Therefore, it was surprising that they are not needed for EBV persistence and even lymphoma formation [32••,33]. In mice transplanted with wt, LMP1 or LMP2 deficient EBV exposed cord blood cells, lymphomas developed at similar frequency. These lymphomas expressed the EBV latency III program and were infiltrated with T cells, irrespective of the presence or absence of LMP1 and 2. Only in LMP1 and LMP2 deficient EBV infections the lymphoma frequency was reduced [33]. Cord blood $CD4^+$ T cells seemed to substitute for the absence of CD40-like signaling in LMP1 deficient tumors, because tumor incidence was reduced

to zero upon $CD4^+$ T cell depletion [32••]. Tumor formation by LMP1 deficient EBV infection could vice versa be restored in $CD4^+$ T cell deficient mice by agonistic CD40 antibody injection [32••]. In contrast, T cell depletion during LMP2 deficient EBV infection did not influence lymphoma formation [33]. These data suggest that cord blood T cells, especially helper $CD4^+$ T cells, support EBV associated lymphoma formation. In contrast, T cell compartments reconstituted from human hematopoietic progenitor cells in HIS mice rather restrict EBV infection and tumorigenesis [18,35,37,38], and these aspects of EBV specific immune control in HIS mice will be discussed next.

4. Modelling EBV specific immune control (500 words)

The common cytokine receptor gamma chain (γ_c) deficiency in the mouse strains that are currently primarily used to generate HIS mice (NSG and BRG) unfortunately compromises IL-7 dependent lymphoid tissue inducer cell development [39]. This results in a paucity of secondary lymphoid tissues, including lymph nodes and mucosal lymphoid tissues, in HIS mice [40]. Therefore, humoral immune responses are severely compromised in HIS mice with circulating IgG serum levels that are thousand fold less than in human peripheral blood serum [41]. However, cell-mediated immune responses can be quite efficiently elicited during EBV infection of HIS mice [42]. Along these lines, primary immunodeficiencies that affect EBV specific immune control mainly compromise cytotoxic lymphocyte responses [9••] and natural killer (NK), $CD4^+$ and $CD8^+$ T cells have been shown to control viral infection and associated tumorigenesis in mice reconstituted with human immune system components from $CD34^+$ hematopoietic progenitor cells [18,26•,35,37,38,43••]. NK cells of HIS mice present after three months of reconstitution from human $CD34^+$ hematopoietic progenitor cells with a phenotype that is quite similar to newborn immune compartments [43••,44]. Immature $NKG2A^+KIR^-$ NK cells expand four weeks after EBV infection of HIS mice and also accumulate in children with infectious mononucleosis [26•,45]. Their depletion increases EBV viral loads tenfold overall and hundredfold in the serum of infected mice starting at week four [26•]. This increased viral load is associated with elevated lymphoma formation. However, NK cell depletion only elevates viral loads and tumorigenesis during wt, but not lytic cycle deficient EBV infection [26•]. Thus, the increased lymphoma formation in NK cell depleted animals seems to be primarily due to increased B cell transformation by new B cell infections. The NK cell compartment of HIS mice can be manipulated to elicit superior immune control of EBV infection. Co-reconstitution of immune system compartments from two donors that are mismatched for the ligands (HLA-B and –C molecules) of inhibitory killer immunoglobulin-like receptors (KIRs) leads to improved suppression of EBV viral loads in the mixed B cell compartments [43••]. This improved immune control most likely results from insufficient KIR mediated inhibition of NK cells from one reconstituted donor by EBV infected B cells of the other donor and vice versa. In addition to NK cells, $V\gamma9V\delta2$ T cells might contribute as cytotoxic innate lymphocytes to the early restriction of EBV infection [46]. Their stimulation with aminobiphosphonate inhibited lymphoma formation during EBV infection of HIS mice. T cells control EBV infection in HIS mice. Primarily $CD8^+$ T cells expand during EBV infection of HIS mice starting at week four after infection, and half of this expansion seems to be due to lytic EBV antigens, because it does not occur during infection with BZLF1 deficient EBV [26•,28,38]. Both individual $CD4^+$ and $CD8^+$ or combined T cell depletion increases viral loads and tumorigenesis, starting at week five after infection

[18,35,37,38]. The signaling lymphocytic activating molecule (SLAM) receptor 2B4, whose activating signaling is compromised in X-linked lymphoproliferative disease type 1 (XLP1) patients that often succumb to primary EBV infection, is required for this T cell mediated immune control in HIS mice [38]. This level of immune control is not present in mice that have received EBV infected cord blood cells [32••,33], but can be somewhat restored by blocking the inhibitory receptors PD-1 and CTLA4 on their T cells [47]. Thus, cytotoxic lymphocytes, whose essential contribution to EBV specific immune control is also identified by primary immunodeficiencies that predispose for EBV associated diseases in humans [9••], mediate immune control of EBV infection in HIS mice. This level of immune control can now be interrogated for contributing T cell specificities, co-receptors on the protective cytotoxic lymphocytes and vaccine formulations that might elicit these protective immune responses.

5. Conclusions and challenges for the future (500 words)

EBV is maybe the only pathogen that has been identified so far to drive an exquisite cytotoxic lymphocyte expansion in HIS mice. This immune response is essential to control EBV infection and associated lymphomagenesis in HIS mice and humans, as identified by the above outlined in vivo studies and primary immunodeficiencies in patients that suffer from EBV associated diseases. We can now interrogate this cytotoxic cell-mediated immune response for its protective value against the different infection programs and associated tumors, which we can modulate by using recombinant EBV viruses and different virus isolates. If it turns out through these studies that particular cytotoxic lymphocytes, including NK and $CD8^+$ T cells, are the protective entities in all these EBV associated disease settings, it becomes important to understand how the human immune system primes such a comprehensive immune control against a pathogen that persistently infects nearly everybody in the adult human population and is one of the most potent cell transforming human pathogens. This is of particular interest because this immune response usually protects us efficiently from the timepoint of primary infection at the age of ten years or younger until the end of our life with sixty or more years. Mimicking such a comprehensive cytotoxic lymphocyte induction by vaccination would not only be beneficial in people, like transplant patients and EBV seronegative adolescents, who are vulnerable to EBV associated diseases, but also against other viral infections and tumors. HIS mice might play an essential role to evaluate the respective vaccine candidates for the induction of such protective cytotoxic lymphocyte responses, initially against challenge by EBV infection.

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•• of outstanding interest

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Figure legend

Figure 1: Lytic replication and immune control of B95-8 EBV infection in HIS mice. Schematic depiction of peripheral blood viral loads during wt (red) and BZLF1 deficient (blue) B95-8 EBV infection (10^5 Raji infectious units) of huNSG mice. Antibody mediated depletion of NK (yellow) and $CD8^+$ T cells (green) during wt B95-8 EBV reveals their role in protection starting at weeks 4 and 5, respectively.